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APPLICATION NUMBER: ***60/545,505***
FILING DATE: ***February 19, 2004***

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.

INVENTOR(S)

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Additional inventors are being named on the 2nd *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

INFLUENZA VACCINE FORMULATED WITH THE NOVEL POLYCATIONIC LIPID D-ERYTHRO CERAMIDE CARBAMOYL SPERMINE (CCS)

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 14 with imbedded drawings
☐ Drawing(s) *Number of Sheets*
☐ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number
☑ Other (specify) transmittal, postcard

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.
☑ A check or money order is enclosed to cover the filing fees.
☑ The Director is herby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 14-0112
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[Page 1 of 2]

Respectfully submitted,

SIGNATURE Lee C. Heiman

Date February 19, 2004

REGISTRATION NO. 41,827 *(if appropriate)*

Docket Number: 26004

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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Docket Number 26004

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Number 2 of 2

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MAIL STOP PROVISIONAL PATENT APPLICATION
Attorney Docket No. 26004

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

BARENHOLZ et al.

Serial No. NOT YET ASSIGNED

Filed: February 19, 2004

For: **INFLUENZA VACCINE FORMULATED WITH THE NOVEL POLYCATIONIC LIPID D-ERYTHRO CERAMIDE CARBAMOYL SPERMINE (CCS)**

TRANSMITTAL LETTER

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Submitted herewith for filing in the U.S. Patent and Trademark Office is the following **PROVISIONAL APPLICATION:**

(1) Transmittal Letter
(2) Cover sheet for filing **Provisional Application**
(3) 14 page Provisional Application consisting of:
14 pages Textual Specification with imbedded drawings,
0 pages of Claims,
0 page of the Abstract,
0 sheets of Drawings;
(4) Check No. 20441 $ 80.00 for filing fee as a small entity;
(5) Postcard for early notification of serial number.

The Commissioner is hereby authorized to charge any deficiency or credit any excess to Deposit Account No. 14-0112.

Respectfully submitted,
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PATENT APPLICATION SERIAL NO. _____

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

02/24/2004 MDANTE1 00000133 60545505
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PTO-1556
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INFLUENZA VACCINE FORMULATED WITH THE NOVEL POLYCATIONIC LIPID D-ERYTHRO CERAMIDE CARBAMOYL SPERMINE (CCS)

In an attempt to further refine the composition of the CCS-flu vaccine, as well as to optimize the mode of application, a series of studies were conducted in mice with the following aims:

1. To compare a single vaccine administration to two administrations (at various time intervals) with regard to immunogenicity and efficacy (Tables 1-3).
2. To test the effect of the antigen dose and the lipid dose on immunogenicity (Tables 4, 5).
3. To test whether the CCS-flu vaccine induces strain cross-reactive antibodies that may afford protection also against heterologous virus strains that are not included in the vaccine (Table 6).
4. To evaluate the CCS lipid assembly as a vaccine carrier for other pathogens (e.g. hepatitis A virus) (Table 7)
5. To analyze the biodistribution of the CCS-based flu vaccine in comparison with flu vaccine formulated with other lipids (Fig. 1).
6. To study the immunomodulatory activity of the vaccine, namely induction of cytokines, nitric acid (NO) and surface molecules (B7, CD40, MHC II) on antigen-presenting cells.

A. CCS-based intranasal (i.n.) trivalent influenza vaccine: comparison of single vs. two administrations

In the experiment described in Tables 1-3, a comparison was made between a single CCS-based vaccine dose (using 2 or 4 μg of antigen [HN] of each viral strain) and two vaccine doses (2 μg/strain/dose), given at 3, 7 or 14 day intervals between administrations. The lipid assemblies were composed of CCS/Chol (cholesterol) at a 3/2 mole ratio, and the lipid/HN w/w ratio was

300/1 for all formulations. As controls, the standard trivalent commercial vaccine (HN) was administered either alone or combined with 1 μg cholera toxin (CT), used as a mucosal adjuvant. Sera, lung homogenates and nasal washes were tested 5-6 weeks after the first vaccine dose for HI antibodies (Table 1), as well as for antigen-specific IgG1, IgG2a, IgA and IgE antibodies (Table 2). In addition, 5 mice from selected groups were challenged i.n. with live virus (using the mouse adapted reassortant X-127 virus) and protection was assessed by quantifying lung virus titer 4 days later (Table 3).

As opposed to the poor or no immunogenicity of the commercial flu vaccine (HN) (groups 2-6), CCS/Chol-flu vaccine induced high titers of all types of antibodies tested (except for IgE which was undetected), especially against the two A virus strains (groups 8-11; Table 1, 2). For the 2-dose regimen, a 1-week interval appears to be the optimal (gr. 10). For the single dose regimen, 4 μg antigen, but not 2 μg (gr. 8 vs. gr. 7), induced high titers of serum and lung HI antibodies, serum IgG1 and IgG2a antibodies and lung IgG1 antibodies. However, in comparison with the 2-dose regimen, the 1-dose regimen did not elicit lung IgG2a and IgA antibodies nor nasal antibodies (Table 2).

In the protection assay (Table 3), the CCS-flu vaccine administered i.n. either once (4 μg) or twice (2 μg/dose) afforded full protection against viral infection (6 log reduction in lung virus titer) whereas the standard vaccine reduced virus titer by only 0.5-1 log. Thus, although the single dose regimen with the CCS-flu vaccine is inferior to the two-dose regimen for certain antibody isotypes, the two regimens provide a similar degree of protection.

In this experiment we also compared CCS alone to CCS/Chol as the vaccine carrier, and found no difference in immunogenicity between the two formulations (data not shown). Another formulation modification was the reduction of the size of the CCS/Chol lipid assemblies (diameter 0.05-5 μm) by extrusion (diameter ≤0.02 μm). Antibody titers induced by the extruded vaccine were 50-80% lower than those produced by the non-extruded vaccine (data not shown). Thus, increasing the antigen dose from 2 μg to 4 μg is critical for the single dose regimen. It is possible that a single i.n. administration of the

CCS-flu vaccine using a higher dose level of both the antigen and the lipid, compared with those used for the two administration regime, would be sufficiently immunogenic.

Table 1

Elicitation of hemagglutination inhibition (HI) antibodies following intranasal vaccination with trivalent influenza vaccine, free and in CCS lipid assemblies, administered once or twice at various time intervals to young (2 mo.) BALB/C mice

No.	Vaccine[a] (n=5)		Dosing days	Mean HI titer (% seroconversion)[b]					
				A/New Caledonia		A/Panama		B/Yamanashi	
				serum	lung	serum	lung	serum	lung
1	None (PBS) x 2		0,7	0	0	0	0	0	0
2	HN 2 μg x1		0	0	0	0	0	0	0
3	4 μg x1		0	0	0	0	0	0	0
4	2 μg x2		0, 3	0	0	0	0	0	0
5	2 μg x2		0, 7	0	0	0	0	0	0
6	2 μg x2		0, 14	0	0	0	0	0	0
7	Lip (CCS/Chol) HN	2 μg x1	0	0	0	0	0	0	0
8	Lipid/HN w/w ratio =	4 μg x1	0	336 (100)	40	328 (100)	40	52 (80)	0
9	300/1	2 μg x2	0, 3	544 (100)	80	408 (100)	40	52 (80)	0
10		2 μg x2	0, 7	544 (100)	80	544 (100)	120	88 (100)	0
11		2 μg x2	0, 14	480 (100)	60	368 (100)	40	80 (80)	0
12	HN 2 μg + CT (1 μg) x2		0, 7	608 (100)	80	664 (100)	120	84 (80)	0

a Mice were immunized with Fluvirin® 2003/2004 trivalent split virion vaccine preparation consisting of A/New Caledonia/20/99 (H1N1)-like, A/Moscow/10/99 (H3N2)-like and B/Hong Kong/330/2001-like, either free (HN) or incorporated into CCS/Chol (3/2 mole ratio) lipid assemblies (0.6 mg for groups 7, 9, 10, 11; 1.2 mg for group 8).

b Serum HI titer was determined on individual mice 5 weeks (day 35) after the first vaccine dose.

Lung (pooled) HI titer was tested on day 42.

In parentheses - % of mice with HI titer ≥40. 0 denotes HI titer <20.

Comment: The commercial trivalent vaccine (Fluvirin®) was concentrated for 13 h. at room temp. x8, using a Speedvac concentrator. This may explain the lower HI titer in this experiment as compared with higher HI titers (1200-2500) obtained in previous experiments with the CCS formulations, using non-concentrated flu vaccine preparations.

Table 2

Elicitation of serum, lung and nasal antigen-specific IgG1, IgG2a and IgA antibodies following intranasal vaccination with trivalent influenza vaccine, free and in CCS lipid assemblies, administered once or twice at various intervals to young (2 mo.) BALB/c mice

No.	Vaccine[a] (n=5)		Dosing days	Mean antibody titer Serum IgG1	Serum IgG2a	Serum IgG2a/IgG1	Serum IgA	Lung Homogenate IgG1	Lung Homogenate IgG2a	Lung Homogenate IgG2a/IgG1	Lung Homogenate IgA	Nasal wash IgG1	Nasal wash IgG2a	Nasal wash IgG2a/IgG1	Nasal wash IgA
1	None (PBS) x 2		0,7	0	0	-	0	0	0	-	0	0	0	0	0
2	HN	2 μg x1	0	0	0	-	0	0	0	-	0	0	0	0	0
3		4 μg x1	0	320	90	0.3	0	1500	0	-	0	0	0	0.	0
4		2 μg x2	0, 3	0	0	-	0	0	0	-	0	0	0	0	0
5		2 μg x2	0, 7	0	0	-	0	0	0	-	0	0	0	0	0
6		2 μg x2	0, 14	40	0	-	0	0	0	-	0	0	0	0	0
7	Lip (CCS/Chol) HN	2 μg x1	0	300	0	-	0	600	0	-	0	0	0	0	0
8	Lipid/HN w/w ratio =	4 μg x1	0	12000	4500	0.4	0	13000	0	-	0	0	0	0	0
9	300/1	2 μg x2	0, 3	15000	10000	0.7	0	15000	2500	0.2	3500	0	10	0	0
10		2 μg x2	0, 7	15000	12000	0.8	0	14000	2500	0.2	9000	200	30	0.15	100
11		2 μg x2	0, 14	13000	5500	0.4	0	12000	1800	0.15	3000	50	0	-	0
12	HN 2 μg + CT (1 μg) x2		0, 7	21000	15000	0.7	0	20000	2500	0.1	2000	250	30	0.1	45

a See table 1 for experimental details. Samples were pooled and tested by ELISA 6 weeks (day 42) after the first vaccine dose. 0 denotes titer <10.

Table 3

Protection of young BALB/c mice against viral challenge following intranasal vaccination with trivalent influenza vaccine, free and in CCS lipid assemblies

No.	Vaccine[a] (n=5)	Dosing days	Lung virus titer (log 10)[b]
1	None	-	6
2	HN 4 μg x1	0	5.5
3	HN 2 μg x2	0, 7	5
4	Lip (CCS/Chol) HN 4 μg x1	0	0
5	Lip (CCS/Chol) HN 2 μg x2	0, 7	0
6	HN 2 μg + CT (1 μg) x2	0, 7	0

a See table 1 for experimental details. In groups 4, 5 the lipid/HN w/w ratio was 300/1.

b The mice were infected intranasally 6 weeks (day 42) after the first vaccine dose, using ~10^6 egg infectious dose 50% (EID 50) of the mouse-adapted reassortant X-127 virus (A/Beijing/262/95 [H1N1] x X-31 [A/Hong Kong/1/68 x A/PR/8/34). Lungs were harvested 4 days later, homogenized, serially diluted, and injected into the allantoic sac of 10 d. fertilized chicken eggs. After 48 h at 37°C and 16 h at 4°C, 0.1 mL of allantoic fluid was removed and checked for viral presence by hemagglutination.

B. The effect of antigen dose and lipid dose on the immunogenicity of intranasal CCS-based flu vaccine

Our standard protocol uses 2 μg antigen/strain/dose and 0.6 mg lipids (lipid/HN w/w ratio = 300/1) for i.n. administration (2 doses spaced 1 week apart). In the experiment described in Tables 4, 5, the trivalent-flu vaccine was formulated with the CCS/Chol lipid assemblies using varying amounts of the HN antigens and the lipid. In this experiment the vaccines were prepared with: (a) varying amounts of the antigen (0.25 - 2 μg) and of the lipid (0.075 - 0.6 mg), keeping the lipid/HN w/w ratio constant at 300/1; (b) graded amounts of the antigen (0.25 - 2 μg) and a constant amount of the lipid (0.6 mg) thereby varying the lipid/HN w/w ratio from 300/1 to 2,400/1. As can be seen in Table 4 (HI titer)

and Table 5 (isotype titers) vaccines prepared at a 300/1 lipid/HN w/w ratio using 2 or 1 μg antigen and 0.6 or 0.3 mg lipid, respectively, produced high and similar levels of antibodies against the 3 viral strains (groups 2, 3). At lower antigen (0.5, 0.25 μg/strain) and lipid (0.15, 0.075 mg) doses the response decreased markedly (groups 4, 5), particularly the mucosal response (lung, nasal) (Table 5). When a constant dose of lipid was used (0.6 mg), high levels of antibodies were obtained even with the two lower doses of antigen (0.25, 0.5 μg/strain) (groups 6-8). Thus, the amount of the lipid is critical, and with the appropriate lipid dose the antigen dose can be reduced 4-8 fold (from 1-2 μg to 0.25 - 0.5 μg).

Table 4

Effect of the antigen dose and lipid dose on the induction of HI antibodies following intranasal vaccination with trivalent influenza vaccine formulated with CCS lipid assemblies, administered twice (at 1 week interval) to young (2 mo.) BALB/c mice

No.	Vaccine[a] (n=5)	HN (μg)	Lipid (mg)	Lipid/HN w/w ratio	Mean HI titer (% seroconversion)					
					A/New Caledonia		A/Panama		B/Yamanashi	
					Serum	Lung	Serum	Lung	Serum	Lung
1	HN	2	-	-	0	0	0	0	0	0
2	Lip (CCS/Chol) HN	2	0.6	300/1	544 (100)	80	544 (100)	120	88 (100)	0
3		1	0.3	300/1	320 (100)	80	544 (100)	160	40 (100)	0
4		0.5	0.15	300/1	416(100)	20	448 (100)	40	32 (100)	0
5		0.25	0.075	300/1	180 (100)	0	100 (100)	20	0	0
6		1	0.6	600/1	672 (100)	80	736 (100)	160	104 (100)	0
7		0.5	0.6	1200/1	560 (100)	80	608 (100	160	104 (100)	0
8		0.25	0.6	2400/1	512 (100)	80	512 (100)	120	48 (100)	0

a See Table 1 for experimental details.

Table 5

Effect of the antigen dose and lipid dose on the induction of serum, lung and nasal antigen-specific IgG1, IgG2a and IgA antibodies following intranasal vaccination with trivalent influenza vaccine formulated with CCS lipid assemblies, administered twice (at 1 week interval) to young BALB/c mice

No.	Vaccine[a] (n=5)	HN (μg)	Lipid (mg)	Lipid/HN w/w ratio	Mean antibody titer											
					Serum				Lung homogenate				Nasal wash			
					IgG1	IgG2a	IgG2a/ IgG1	IgA	IgG1	IgG2a	IgG2a/ IgG1	IgA	IgG1	IgG2a	IgG2a/ IgG1	IgA
1	HN	2	-	-	0	0	-	0	0	0	-	0	0	0	-	0
2	Lip (CCS/Chol) HN	2	0.6	300/1	15000	12000	0.8	0	14000	2500	0.2	9000	200	30	0.15	100
3		1	0.3	300/1	14000	2500	0.2	0	10000	1000	0.1	8000	100	0	-	80
4		0.5	0.15	300/1	15000	1300	0.1	0	8000	1500	0.2	4000	0	0	-	0
5		0.25	0.075	300/1	12000	400	-	0	3500	400	0.1	2500	0	0	-	0
6		1	0.6	600/1	20000	15000	0.8	0	12000	2500	0.2	8000	200	15	0.1	80
7		0.5	0.6	1200/1	15000	14000	0.9	0	15000	5000	0.3	15000	150	35	0.2	100
8		0.25	0.6	2400/1	15000	9000	0.6	0	21000	2500	0.1	13000	250	25	0.1	90

a See Tables 1, 2 for experimental details.

C. CCS-based vaccine induces high titer of strain cross-reactive HI antibodies

The data shown in Table 6 indicate that intranasal (i.n.) and intramuscular (i.m.) vaccination, administered once or twice, with either a monovalent or trivalent CCS-based influenza vaccine elicits high serum titers of HI antibodies directed against the immunizing strains and cross-reacting with several A/H1N1, A/H3N2 and B strains that were circulating in the years 1986-1999 and were not included in the vaccine. Slightly lower HI titer were found after a single i.n. vaccine dose (gr. 6 vs. gr. 7). Lung homogenate HI titers (gr. 4, 8) were lower than the corresponding serum titers. Thus, parenteral or intranasal vaccination with the CCS-based vaccine may afford protection against a wide spectrum of A and B viral strains. Such antigenic variants may emerge during a flu epidemic as a result of antigenic drift. In contrast, the standard commercial vaccine administered i.n. (gr. 1, 5) was totally ineffective in inducing antibodies against both the homologous and the heterologous strains.

Table 6

Induction of strain cross-reactive HI antibodies following intranasal or intramuscular vaccination of young BALB/c mice with CCS-based monovalent and trivalent influenza vaccine

No.	Vaccine[a]	Vaccine strains	Sample tested	Mean HI titer against: A/H1N1 New Caledonia/ 20/99	Beijing/ 262/95	Texas/ 36/91	Singapore/ 6/86	A/H3N2 Panama/ 2007/99	Sydney/ 5/97	Nanchang/ 333/95	Johannesburg/ 33/94	B Yamanashi/ 166/98	Harbin/ 07/94
1	HN 2 µg x2 i.n.	A/New Caledonia	serum	0	0	0	0	0	0	0	0	0	0
2	Lip HN 2 µg x2 i.n.		serum	1280	1280	1280	240	0	0	0	0	0	0
3	Lip HN 1 µg x1 i.m.		serum	640	640	320	40	0	0	0	0	0	0
4	Lip HN 2 µg x2 i.n.		lung homo-genate	320	240	240	20	0	0	0	0	0	0
5	HN 2 µg x2 i.n.	A/New Caledonia, A/Panama, B/Hong Kong	serum	0	0	0	0	0	0	0	0	0	0
6	Lip HN 4 µg x1 i.n.		serum	320	80	120	0	320	320	120	120	60	120
7	Lip HN 2 µg x2 i.n.		serum	480	120	240	20	640	640	120	120	80	320
8	Lip HN 2 µg x2 i.n.		lung homo-genate	80	80	40	0	120	80	0	0	0	40
9	HN 2 µg + CT 1 µg x2 i.n.		serum	480	240	120	40	480	480	120	120	80	240

a Pooled sera and lung homogenate obtained 4-6 weeks after vaccination were tested for HI antibodies. For experimental details, see Table 1. The lipid (Lip) assemblies were composed of CCS/Chol (3/2 mole ratio) and the lipid/HN w/w ratio was 300/1. Except for groups 3 and 6, the two vaccine doses were spaced 1 week apart. 0 denotes HI titer <20.

D. Induction of mucosal response against hepatitis A virus (HAV) with CCS liposomes

In addition to influenza, the immune enhancing potential of CCS liposomes was also tested for HAV vaccine administered by various mucosal routes. The data presented in Table 7 show that whereas the commercial HAV vaccine failed to induce an IgA response in both tissues (lamina propria, Peyer's patches) tested, and by both administration routes (i.n., i.r.), the vaccine formulated with either the CCS liposomes or CpG-ODN generated a significant response in most cases. The combination of HAV-CCS liposomes and CpG-ODN resulted in a synergistic response in all cases. Thus, CCS liposomes alone, and particularly in combination with CpG-ODN, are also effective as a carrier/adjuvant for mucosal vaccination against HAV.

Table 7

Induction of IgA antibodies following intranasal (i.n.) or intrarectal (i.r.) vaccination of BALB/c mice with hepatitis A virus (HAV) vaccine, alone and in combination with CCS liposomes and/or CpG-ODN

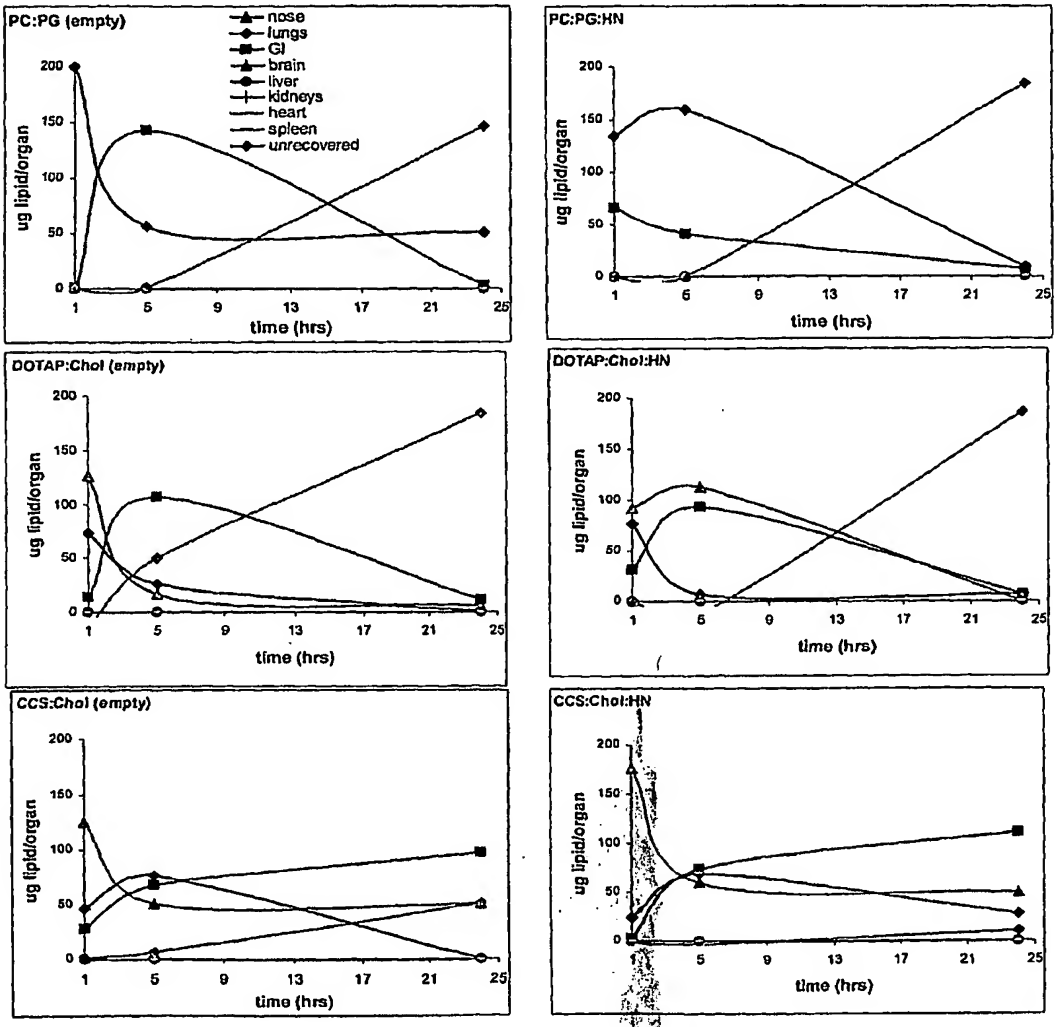
Vaccine	Mean no. of IgA AFC/10^6 cells in:			
	Lamina propria		Peyer's patches	
	i.n.	i.r.	i.n.	i.r.
HAV alone	0	0	0	0
HAV-CCS liposomes	12	27	0	1
HAV + CpG-ODN	16	22	0	14
HAV-CCS liposomes + CpG-ODN	139	68	28	23

HAV vaccine (Aventis Pasteur), 10 EU (~1.5 µg protein), was administered twice at a 2-week interval and the response was tested by the ELISPOT technique 3 weeks after the second vaccine dose. CpG-ODN, used as a mucosal adjuvant, was given at 10 µg/dose. The HAV-CCS liposomes were prepared as described above for the influenza vaccine (Table 1).
AFC – antibody-forming-cells

E. Biodistribution of CCS liposomes administered intranasally

In this biodistribution experiment, 3 formulations of lipid-labeled liposomes: DMPC/DMPG (anionic), DOTAP/Chol (cationic) and CCS/Chol (cationic), either empty or loaded with the influenza HN antigens, were administered intranasally (200µg lipid per mouse) into BALB/c mice. The fluorescently labeled lipid was then traced in the homogenates of various tissues over a period of 24 h. As can be seen in Fig 1, the CCS formulation containing the HN antigens displayed the longest retention (>24h.) in the 3 target organs (nose, lungs, GI tract) while there was no lipid accumulation in the brain and no significant accumulation in the other organs tested (liver, kidneys, heart, spleen). This long retention of the CCS vaccine in the respiratory and GI tracts may explain its superior immunogenicity over the other liposomal formulations.

Fig 1
Lipid biodistribution of various liposomal formulations, with and without the influenza HN antigens, following intranasal administration to mice



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F. Preliminary studies on the immunomodulatory activity of CCS-flu vaccine in mice

In these experiments, mice were injected i.p. with various liposomal formulations (composed of DMPC, DMPC/DMPG, DOTAP/Chol, CCS/Chol), 0.5-1 mg lipid, with or without the HN antigens. The mice were either untreated or i.p. injected with thioglycollate (TG, to increase macrophage production) 2 days before the injection of the liposomal formulations. Peritoneal cells were harvested 24-48 h. after administration of the liposomes and used as such or after 4 h. adsorption at 37°C to plastic dishes and removal of the non-adherent cells. In other experiments, peritoneal cells were harvested from TG treated mice and incubated with the liposomal formulations for 24-48h. The cells were tested by flow cytometery for the expression of MHC II and the co-stimulatory molecules CD40 and B7. The supernatants were tested for the cytokines interferon γ (IFN γ), tumor necrosis factor α (TNF α) and interleukin 12 (IL-12), and for nitric oxide (NO).

All the cationic formulations (CCS/Chol, DOTAP/Chol, DMTAP/Chol) upregulated the expression of B7 and CD40 more than the other formulations (DMPC [neutral], DMPC/DMPG [anionic]) and induced higher levels of IFN γ and IL-12. In some cases the CCS/Chol formulation was more effective than the other cationic formulations. No significant levels of TNF α and NO were induced by any of the formulations. The upregulation of the co-stimulatory molecules and the induction of IL-12 and IFN γ by the cationic formulations can explain, in part, the greater adjuvant activity of these formulations. These findings combined with the long retention of the CCS-flu vaccine in the respiratory tract (Fig. 1) after intranasal administration may shed light on the mechanism of action of CCS as an efficient mucosal vaccine carrier/adjuvant.

PCT/IL 2004/000536

03 AUG 2004

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THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

June 28, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: *60/537,553*
FILING DATE: *January 21, 2004*

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SUBMITTED OR TRANSMITTED IN
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COVER SHEET FOR FILING U.S. PROVISIONAL APPLICATION UNDER 37 CFR 1.53(c)

Commissioner of Patents and Trademarks
Alexandria, Virginia 22313-1450

Re: New U.S. Provisional Patent Application
For: **THERAPEUTIC USES OF POLYCATIONIC CERAMIDES**
Inventors: Yechezkel BARENHOLZ; Jerusalem, ISRAEL
Attorney Docket: 25951

Sir:

Attached hereto is the application identified above, including:

24 Pages Application Consisting of:
24 Pages of Textual Specification
0 Pages of 0 claims
0 Pages containing the Abstract of the Disclosure
0 Pages of Drawings
__ Executed Inventor's Declaration

The present provisional application names the following inventor(s): 1) Yechezkel BARENHOLZ; Jerusalem, ISRAEL

The Government filing fee* is calculated as follows:
Base Fee (Provisional Application) . . . . . $ 160.00

TOTAL FILING FEE*
(accounting for possible small entity status) . . . $ 80.00

X *Reduced by one-half, as applicant(s) is/are a "small entity".

0 Sheets of Drawing(s) is/are attached.

X Submitted herewith is a check in the amount of $ 80.00. The Commissioner is hereby authorized to charge any deficiency or credit any excess to Deposit Account No. 14-0112.

Respectfully submitted,

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GMN/LCH/ls (ProvisionalAppl.coversheet)

MAIL STOP PROVISIONAL PATENT APPLICATION
Attorney Docket No. 25951

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Yechezkel BARENHOLZ

Serial No. Not yet assigned

Filed: January 21, 2004

Title: **THERAPEUTIC USES OF POLYCATIONIC CERAMIDES**

TRANSMITTAL LETTER

The Commissioner for Patents
Alexandria, Virginia 22313-1450

Sir:

Submitted herewith for filing in the U.S. Patent and Trademark Office is the following **PROVISIONAL APPLICATION:**

(1) Transmittal Letter
(2) Cover sheet for filing Provisional Application
(3) 24 page Provisional Application consisting of:
24 pages Textual Specification
0 pages of 0 claims
0 pages containing the Abstract of the Disclosure
0 sheets of drawings
(4) Check No. 20223 $ 80.00 for filing fee
(5) Postcard for early notification of serial number.

The Commissioner is hereby authorized to charge any deficiency or credit any excess to Deposit Account No. 14-0112.

Respectfully submitted,

NATH & ASSOCIATES PLLC

By: _____

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PATENT APPLICATION SERIAL NO. _____

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PTO-1556
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Therapeutic uses of polycationic ceramides

Determination of cytotoxicity of various lipoplexes containing anti Bcl-2 siRNA against MCF-7 tumor cells by methylene blue (MB) staining assay.

siRNS is small interference RNA which acts like knockout and silences gene expression. The biological experiments described herein were performed on tumor cells having high expression of the Bcl2 protein which is known to inhibit apoptosis of said tumor cells. Thus, by silencing Bcl2 gene expression the level of the protein is reduced and program cell death is initiated, resulting in a low survival rate of the tumor cells. The results presented herein show very clearly that the cationic lipids are efficient in delivering siRNA in superiority to commericially available delivery systems (e.g. sifect and mirus system)

The experiments:

- MCF-7 cells were plated into 96 well plate at density of $4*10^3$ cells/well and allowed to grow for 24 hr.
- After indicated time period, various lipoplexes at the cationic lipid/siRNA P^- ratio of 2/1 were added into the cells and incubated for 72 hr. Two different types of siRNA (Dharmacon, USA) were used:

  siRNA I (19 nucleotides, MW=13.600)
  siRNA II (22 nucleotides, MW=14.025)

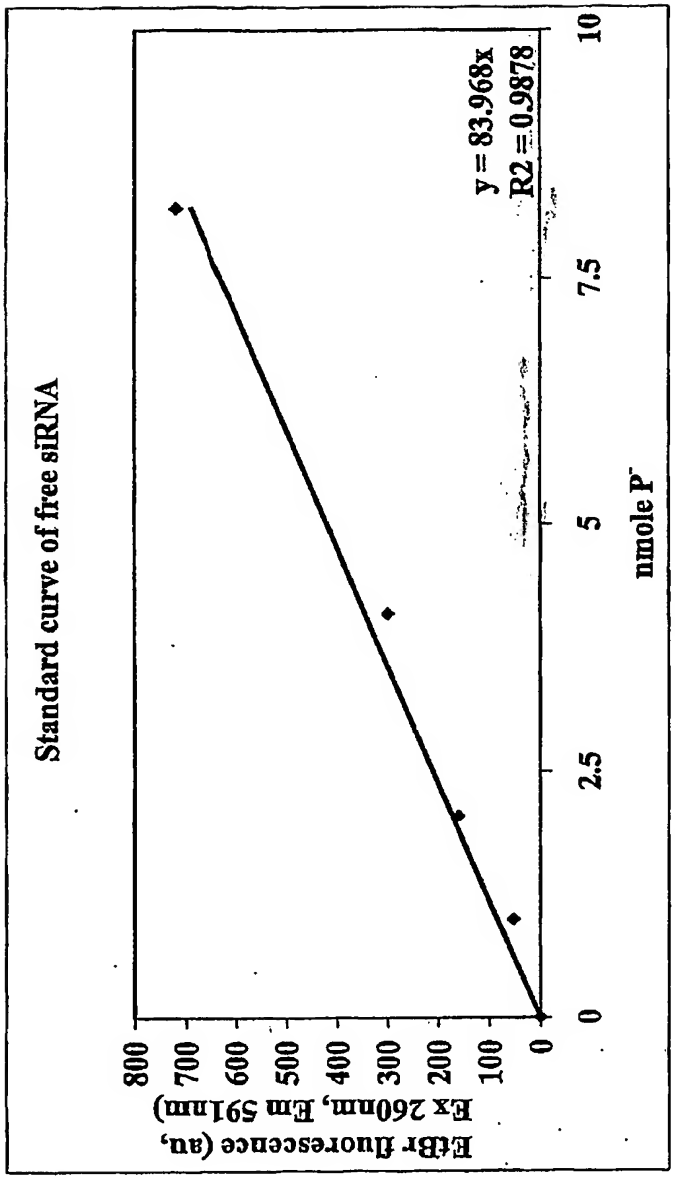
Additional controls include:

- Comparison with the carriers TransIT-TKO (Mirus, USA) and siFect^{tm} (Promega, USA) commercial kits.
- 18 mer G3139 Bcl-2 ODN antisense (Genta, Lexington, Ma)
- Scrambled antisense
- Rabies virus siRNA

3. Following 72 hr of growth, cells were fixed by 2.5% glutaraldehyde and stained with MB. The MB bound to the fixed cells was extracted by 0.1 N HCl, and the net optical density of the dye in each well was determined by a plate spectrophotometer (Labsystems Multyskan Bichromatic, Finland) at 620 nm.

Anti-Bcl-2 si RNA binding to DOTAP and CCS based lipoplexes

Cationic liposome formulations	Type of siRNA	siRNA nmole P^- /cationic lipid ratio	Free siRNA(%)
CCS	I	1/2	0
CCS	II	1/2	0
DOTAP	I	1/2	0
DOTAP	II	1/2	14
CCS/DOPE (2:1)	I	1/2	0
CCS/DOPE (2:1)	II	1/2	0
DOTAP/DOPE (2:1)	I	1/2	0
DOTAP/DOPE (2:1)	II	1/2	15
CCS/Cholesterol (2:1)	I	1/2	0
CCS/Cholesterol (2:1)	II	1/2	0
DOTAP/Cholesterol(2:1)	I	1/2	20
DOTAP/Cholesterol (2:1)	II	1/2	15.5



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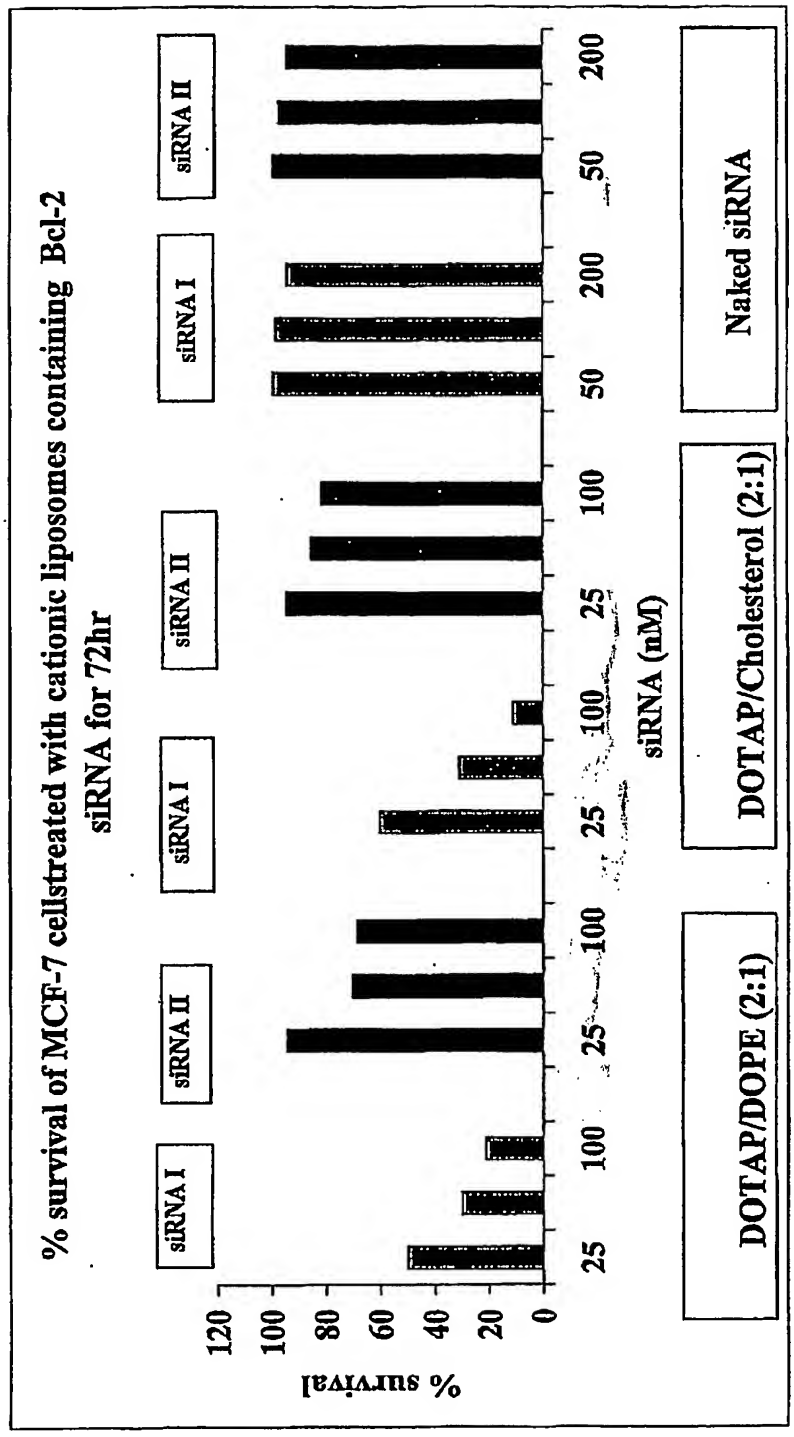

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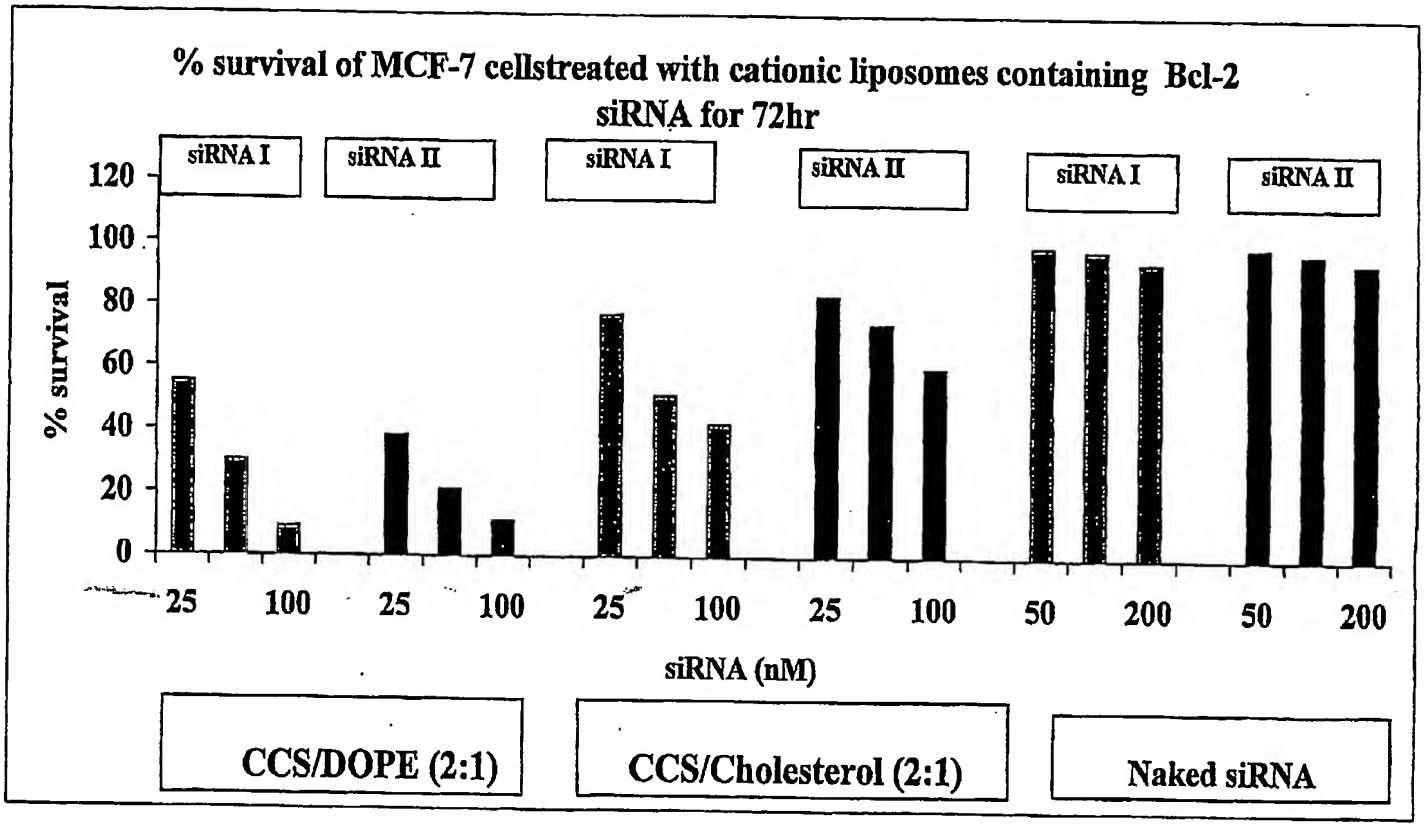

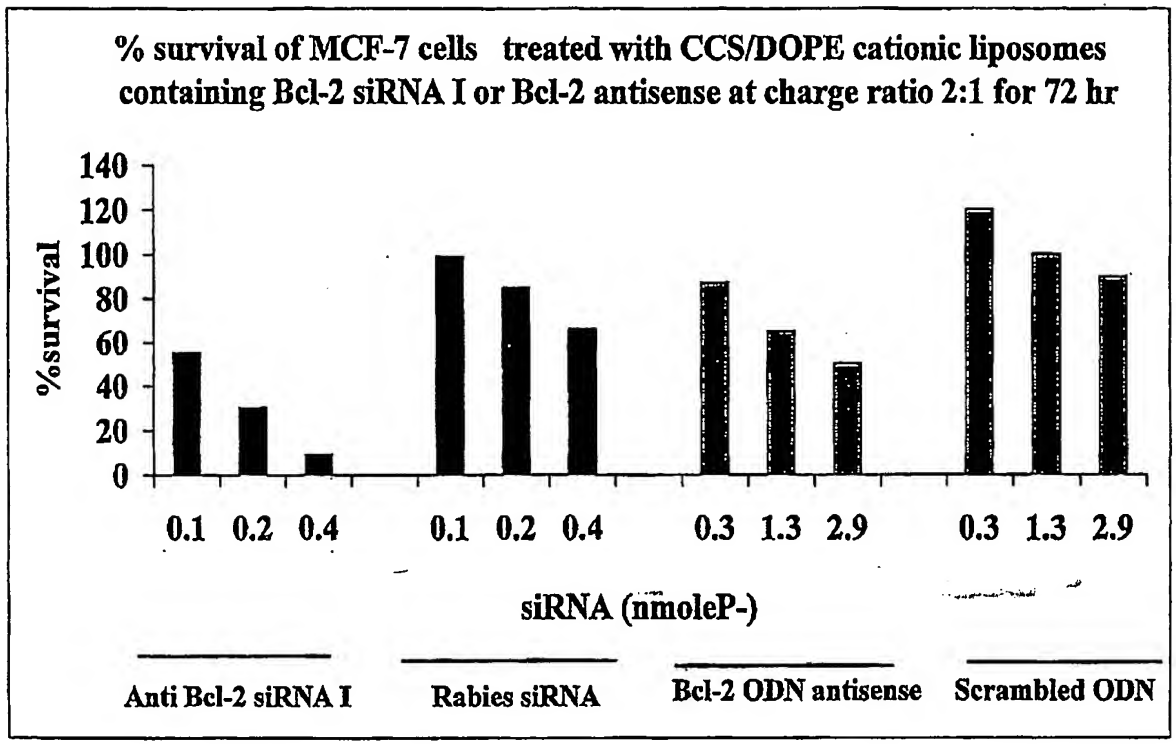

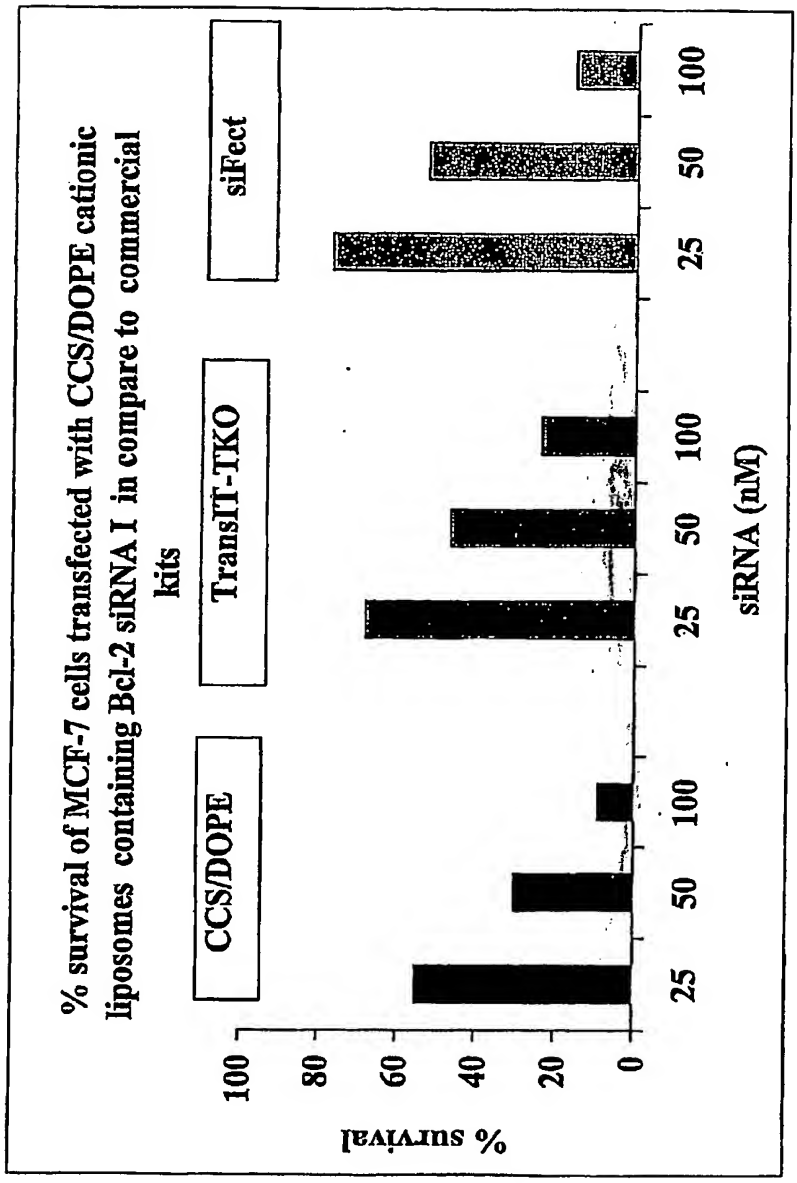

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B Background and Significance

B1 Background

B1.1 Influenza – the disease and the disadvantages of the available vaccins: Influenza is a significant cause of morbidity and mortality, and flu viruses are considered a potential bio-threat (NIAID category C). Rates of infection are highest among children, but rates of serious illness and death are highest among individuals aged ≥65 years and persons of any age with underlying medical conditions that put them at higher risk for influenza complications [6a]. It is estimated that the average annual global burden of inter-pandemic influenza may be in the order of 1 billion (~20% of the population) cases of flu, ~3-5 million cases of severe illness, and 250-500,000 deaths. Global pandemics occur at unpredictable intervals (10-30+ years) resulting from antigenic shifts causing the emergence of completely new flu virus subtypes, for which there is no existing immunity in the human population. The 3 major pandemics in the 20th century (1918, 1957 & 1968) caused a total of 40-80 million deaths worldwide. The global impact of the next pandemic is projected to be 1-2 billion cases of flu, 5.3-12.3 million cases of severe illness, and 1.5-3.5 million deaths [2-6]. Flu epidemics typically occur during the winter months and were responsible for some 36,000 deaths/year on average in the USA from 1990-99 [6a].
Annual vaccination with inactivated flu vaccines administered i.m. effectively prevent illness in 70-90% of healthy adults, but in the elderly (over 65) vaccination efficacy is 50% or less. Furthermore, current influenza vaccines are underutilized: only 32% of the U.S. adults (18-64 y) with high-risk conditions are immunized. The WHO estimates that there are about 1.2 billion people at "high-risk" for severe influenza outcomes [2-6a]. In view of the relatively low efficacy of the current flu vaccines in the major high-risk groups, improvement of the efficacy of the available vaccines, and development of novel, more effective vaccines targeting high-risk groups (infants, immunodeficient patients and especially the elderly), remains a high priority.

B1.2 Current flu vaccines: The most dominant flu vaccines now in use are composed either of trivalent split virion or subunit preparation without any adjuvant and designed for i.m. administration. Alum, the adjuvant approved for most anti-pathogen vaccines, is not effective in the case of flu vaccines.

B1.3 Why mucosal (intranasal) vaccines? Although most pathogens use mucosal routes (e.g., respiratory, gastrointestinal, urogenital) for invasion, the majority of the currently available vaccines are administered intramuscularly (i.m.). Intramuscular immunization is very effective in evoking a systemic immune response; however, local immunity at the portals of pathogen entry is often suboptimal. In contrast, potent mucosal vaccines (i.e., those administered intranasally or orally) can trigger both local and systemic immunity, thus aborting the primary infection and its accompanying morbidity. Additional advantages of mucosal vaccines over injectable vaccines are: 1) vaccine delivery is simple and painless, and avoids the risk of infection from recycled needles and syringes, which may be relevant in some developing countries; 2) needle-free vaccines would increase public participation in vaccination; 3) since such vaccines can be self-administered, cost-effectiveness may be increased by reducing physician and healthcare staff expenses; 4) large populations can be immunized within a short period of time, which is critical in cases of sudden worldwide pandemics or a bioterror attack; 5) systemic adverse reactions may be reduced, and 6) the mucosal immune system may be less affected by aging and other immunocompromising conditions than the systemic immune system [7-9].

To date, oral vaccination has been problematic due to the degradation of the vaccine components in the gastrointestinal system and the tolerogenic environment in this compartment. Therefore, the intranasal (i.n.) route appears to be preferable. However, even i.n. vaccines require powerful immunoadjuvants in order to be effective [8,9]. The most potent mucosal adjuvants known today are the enterotoxins derived from Vibrio cholerae (cholera toxin, CT) and the heat-labile toxin (LT) from E. coli [10-12]. Yet, because of their toxicity, these agents are not approved for human use [12a]. Even

attenuated versions of these toxins can cause adverse reactions in humans. In this regard, an intranasal flu vaccine ("NasalFlu"), which contains a "non-virul nt" LT, made by Berna Biotech, Bern, Switzerland, was withdrawn in 2002 due to n urological advers reactions [13]. Thus, mucosal vaccines should either contain no adjuvants or be supplemented with potent, but safer adjuvants, for example, synthetic oligodeoxynucleotides containing unmethylated CpG (cytosine-phosphate-guanine) motifs (CpG-ODN) [14]. Liposomes and other lipid-based assemblies are a promising delivery system/adjuvant for mucosal vaccines. Our own studies [15-21], and those of others [22-24], have demonstrated that liposome-encapsulated vaccines are more effective than free, non-liposomal vaccines in both animals and humans, when administered parenterally (e.g., i.m., i.p., s.c) or locally (i.n.) (See B1.5).

B1.4 Novel "state of the art" flu vaccines: Several new-generation flu vaccines have recently been approved for human use [3-5, 25]. A European-licensed "virosomal" flu vaccine (**Inflexal-v**, Berna Biotech Ltd., Bern, Switzerland), which is a subunit vaccine encapsulated in liposomes and injected i.m., has at least the same immunogenicity as, and possibly less reactogenicity than, the standard vaccine. **FluAd** is an injectable flu vaccine formulated in MF59 (an oil-in-water emulsion) as an adjuvant (Chiron Vaccines, USA). This vaccine (licensed in Europe) modestly increases (~1.5-2-fold) the geometric mean serum HI antibody titer in young adults compared to the standard vaccine, while being less effective in the elderly, and it is mildly reactogenic. An intranasal virosomal vaccine adjuvanated with an attenuated version of the E. Coli LT (**NasalFlu**, Berna Biotech) has been reported to elicit both nasal IgA and systemic IgG antibodies [26]. However, following licensing in 2001 it was withdrawn from the market by the manufacturer due to several cases of transient Bell's palsy (facial paralysis), in addition to other local and systemic adverse reactions attributed mainly to the LT adjuvant [12a]. Recently, a cold-adapted, live-attenuated vaccine (**FluMist**, MedImmune Vaccines, Inc., USA) for intranasal administration has been approved by the FDA for healthy individuals of the age group of 5-49 years, excluding several high-risk groups, including infants/toddlers and the elderly, immunodeficient patients, pregnant women, and patients suffering from various chronic respiratory, cardiovascular and metabolic diseases [27]. Furthermore, this vaccine requires freezing for storage, trained medical personnel for delivery, and it is ca. 4 times more expensive than the standard vaccine.
Another intranasal flu vaccine in development is **FluInsure** (ID Biomedical Corp, Canada), which is a subunit vaccine formulated with proteosomes and is administered in one or two doses [28]. Recent phase II clinical trials have demonstrated high efficacy and good tolerability. However, since the proteosome formulation consists of the outer membrane proteins of bacteria in which the flu virus antigens are incorporated, and bacterial proteins are immunogenic in humans, repeated annual vaccination with proteosomes may generate a high titer of neutralizing antibodies against proteosome proteins. Such antibodies may incapacitate the proteosomes' ability to induce an anti-influenza immune response. In contrast, our vaccine carrier is made of lipids (see below), which are considered to be poorly or non-immunogenic, therefore no such neutralizing antibodies against the vaccine carrier are expected.

B1.5 Our approach, based on a novel polycationic lipid as a vaccine carrier/adjuvant

Liposomes (microscopic, synthetic lipid vesicles, with a diameter of 20 nm-10μm) and lipid assemblies alike, including several anti-cancer and anti-fungal drugs based on lipid assemblies, are already widely used [29]. The advantages of such systems as a delivery system for biologicals (i.e., vaccines) and drugs over other delivery systems or the administration of the agent in free, soluble form are [22-24, 30, 31]: 1) high encapsulation efficiency of biologicals and drugs under relatively mild conditions; 2) versatility in composition, structure (liposomes, micelles, lipoplexes, other assemblies), size (from 20 nm to μm range), lamellarity, and electrical charge that can be tailored according to the specific application; 3) biodegradability, biocompatibility, poor or no immunogenicity, and good safety profile in animals and humans upon administration by various routes; 4) while in the xtracellular space, the entrapped/associated agent is

protected from th deleterious effects of enzymes or antibodies; 5) th slow release of the entrapped agent (depot effect) over an extended period of time, and the possibility of targ ting lipidic assemblies to various cells and tissues, thereby increasing agent level in target organs and reducing toxicity to normal tissues; 6) th inherent immunostimulatory activity of liposomes and lipid assemblies alike, resulting from their selectiv accumulation in the lymphoid organs and tissues and efficient uptake by antigen-presenting cells; 7) In view of 4)-6), the possibility of reducing agent dose and number and frequency of administrations; and 8) the vast academic and practical/applied knowledge available, and the ability of mass-production under GMP or GLP conditions [22-24, 29-32]. The advantage of such lipid assemblies as an antigen carrier is that it is composed of low molecular weight, fully synthetic molecules which are self assembled into a supramolecular assembly. This is attractive as a pharmaceutical and does not suffer from problems that affect proteins and other macromolecules originating from live organisms.

As indicated above, a major problem with mucosal vaccines, particularly those consisting of killed microorganisms or their purified products, is poor immunogenicity. To date, most liposomal vaccine formulations have been designed for parenteral administration and are composed of neutral or negatively-charged (anionic) lipids. Such formulations are usually inefficient when administered via the mucosa. Two injectable (i.m.) **virosomal** vaccine formulations, against hepatitis A [33] and influenza [34], are already in use in humans. Recently, we have found that certain formulations of cationic liposomes, comprising the commercially available monocationic lipids DOTAP or DMTAP, or the micelles/lipid assemblies based on the novel polycationic lipid D-erythro ceramide carbamoyl-spermine (**CCS** - see Fig. 1 for formula), are powerful delivery systems for intranasal flu vaccines in mice (see Section C). The mucosal (nasal, lung) and systemic (serum, spleen) humoral and cellular responses obtained were up to 10,000 times greater than those achieved with the commercial influenza vaccine. Cationic liposomes are widely used both in animals and in patients in gene transfection, gene therapy and genetic vaccination [35-37]. Their positive charge allows for efficient electrostatic association/complexation with both nucleic acids and the cell membrane, followed by internalization through adsorptive endocytosis [36, 38]. In contrast, very few studies have been carried out so far with cationic liposomes as a carrier of protein antigens rather than antigen-encoding genes. These studies already indicated the potential of antigen-laden cationic liposomes as efficient parenteral and mucosal vaccines in animals [39-42], including an experimental intranasal flu vaccine using liposomes consisting of DC-Chol that was developed by Aventis Pasteur [42]. Interestingly, this particular formulation was the least effective of all the cationic formulations we screened (Section C, Figs. 4-6).

In the proposed project, we plan to further develop and optimize the cationic lipid formulations, particularly the CCS-based formulations, as a carrier/adjuvant for novel i.n. vaccines against **influenza** that will replace or supplement the currently used vaccines.

B.2 SIGNIFICANCE

Efficacious, safe mucosal vaccines that can be used in mass vaccination within a short period of time are in great need for: (a) controlling pandemic, fast-spreading diseases such as Influenza and SARS; (b) "mucosal" pathogens (e.g., HIV, M. Tuberculosis) for which no effective vaccines exist; and (c) bioterror agents. Such vaccines may also supplement or replace existing vaccines. With regard to influenza, the available parenteral vaccines do not provide sufficient protection for the elderly; and the novel, intranasal "**FluMist**" vaccine is at present approved only for healthy individuals of the age group 5-49 years-old, thus excluding almost all the high-risk groups.

Our novel, proprietary polycationic lipid CCS is apparently non-toxic (shown in mice), easy to prepare even by lay persons (simple mixing of the dried lipid with the standard vaccine) and can be self-administered (by adults). It can be applied to the entire population including high-risk groups and is also effective without an exogenous (and potentially toxic) adjuvant. These factors make this novel carrier/adjuvant a promising platform for new-generation flu vaccines, as well as for other mucosal pathogens. The proposed mechanistic studies are aimed at clarifying why certain synthetic cationic

lipids are such strong activators of immune response (e.g., whether they act as "danger" signals), and will enable rational optimization of the CCS formulation. The proposed clinical trials ar expected to provide preliminary information regarding the safety and effectiveness of the i.n. CCS-flu vaccine in comparison with the standard i.m. vaccine.
The extended preliminary results with the CCS-flu vaccine (C 3) and the preliminary data achieved with the CCS-based *C. botulinum* toxoid vaccine (Fig. 9), as w ll as results achieved with a CCS-based *paratuberculosis* vaccine (data not shown) suggest that CCS (with/out helper lipids) may serve as a platform for various i.n. vaccines. Such vaccines may have similar advantages to those described in B1.5 above.

C. Preliminary Studies

Protective immunity to influenza virus infection is mediated by: (1) IgA antibodies (Abs) in the respiratory tract; (2) serum and mucosal IgG Abs; and (c) cytotoxic T lymphocytes (CTL). Both anti-hemagglutinin (HA) and anti-neuraminidase (NA) antibodies play a role in protection [9, 42a, 42b]. Therefore, we evaluated the efficacy of our novel liposomal influenza vaccines for their ability to elicit anti-HA and anti-NA Abs, mucosal IgA Abs, IgG1 and IgG2a Abs (indicating Th2 [humoral response] and Th1 [cellular response], respectively), CTL, and protective immunity to virus challenge.

C.1 First-generation liposomal vaccines

The extensive collaboration between Y. Barenholz (YB) and E. Kedar (EK) over the past 12 years has resulted in the production of several **liposomal** formulations consisting mainly of neutral (DMPC) or anionic (DMPC/DMPG) lipids, in which the influenza, hepatitis B and M. tuberculosis antigens, as well as several immunoadjuvants (IL-2, GM-CSF, IFNγ, CpG-ODN), were incorporated with high efficiency (60-90%) in the same or in separate vesicles. Such formulations were found safe and much more effective than the non-liposomal formulations upon i.m., s.c. and i.p. administration to young, old and immunosuppressed mice [15-19]. We tested one such product, an injectable (i.m.) liposomal influenza (subunit or split virion) vaccine supplemented with IL-2 as an adjuvant (designated **INFLUSOME-VAC**), in 2 randomized clinical trials at the Hadassah Medical Center, Jerusalem in 2000-01. In a study of 53 healthy young adults (mean age 28 y), INFLUSOME-VAC was significantly ($p<0.05$) more effective than the standard vaccine against all 3 viruses included in the vaccine [20]. In a second trial in community-residing elderly volunteers (mean age 81 y), 81 individuals were vaccinated either with the standard vaccine (n=33) or with INFLUSOME-VAC (n=48). At 1 month post-vaccination, seroconversion (4-fold rise in HI titers) rates for the A/New Caledonia (H1N1) and A/Moscow (H3N2) strains were significantly higher ($p = 0.04$) in the INFLUSOME-VAC group (65 vs. 45%, 44 vs. 24%, respectively). Moreover, INFLUSOME-VAC induced a greater anti-neuraminidase (NA-N2) response ($p<0.05$). Adverse reactions were similar in both groups [21]. Thus, INFLUSOME-VAC appears to be both safe and more immunogenic than the currently used vaccine, even in the elderly. The high titers of anti-neuraminidase Abs elicited by INFLUSOME-VAC, but not by the standard vaccine, in both mice and humans, is of particular interest, because the antigenicity of this molecule is less variable than that of hemagglutinin. Such Abs can provide protection to a wide spectrum of virus substrains [42b] as we demonstrated in mice [17].
Over the past 4 years YB and EK's collaboration has been focused on the studies described below which serve as the basis for this proposed project.

C.2 Second-generation liposomal vaccines based on CpG-ODN as an adjuvant: Since INFLUSOME-VAC was found to be relatively ineffective upon i.n. administration in mice, we replaced cytokines with CpG-ODN [14] as a mucosal adjuvant, using the same lipid composition. Vaccination (i.n. or i.m.) with the standard influenza and hepatitis B vaccines in conjunction with liposomal CpG-ODN (ODN 1018) markedly enhanced (up to 20-fold) the humoral response (serum, nasal and lung IgG1, IgG2a, IgA) and cellular response (proliferation, cytotoxicity, cytokine production) compared with the responses obtained with antigen alone or antigen + free CpG-ODN

[19]. The IgG2a and IgA responses obtained with liposomal CpG-ODN were similar to those obtained when cholera toxin (CT) was used as an adjuvant; however, protection following virus challenge was somewhat better with liposomal CpG-ODN [19]. However, this INFLUSOME-VAC containing CpG-ODN did not meet all the expectations of an i.n. vaccine, especially in the protection-challenge experiments and in the local immune responses (lung and nasal IgA and IgG) that were not high enough [19]. This led to the development of a third-generation cationic lipid-based vaccine for i.n. administration.

C.3 **Third-generation: cationic lipid-based intranasal vaccines.** Due to the poor performance of the neutral (DMPC) and anionic (DMPC/DMPG) liposomes as antigen carriers for intranasal vaccination, we have recently developed and tested the "third-generation" lipid-based vaccines consisting of both commercially available monocationic lipids (e.g., DSTAP, DMTAP, DOTAP) and the novel, fully synthetic polycationic sphingolipid having the natural stereochemistry of D-erythro ceramide-carbamoyl spermine, **CCS** [jointly developed by the PI (YB) and Dr. E. Rochlin (BioLab Ltd., Jerusalem), provisional patent application filed June 2003].

The CCS formula is presented in Fig.1. The cryo-transmission electron microscope (TEM) image of the CCS/Cholesterol (Chol) (3:2 mole ratio) formulation is shown in Fig.2. Vesicles of varying diameter (0.05-3 μm) and shapes (small liposomes, large multilamellar lipid assemblies) are demonstrated.

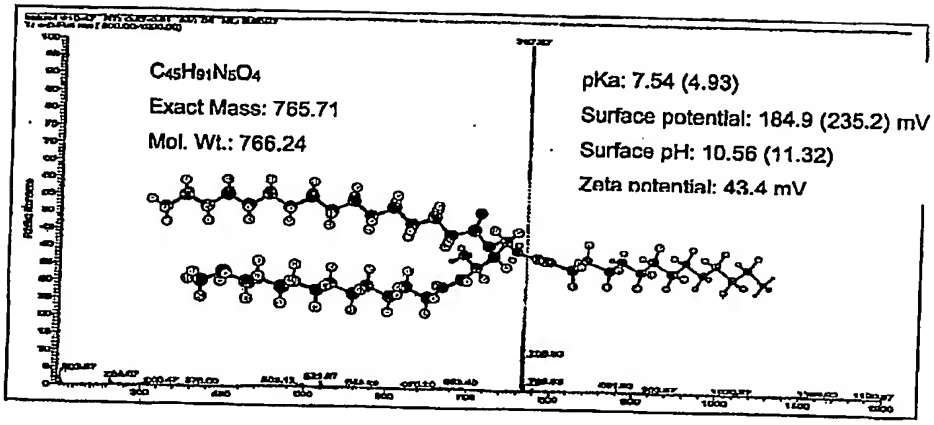


Fig. 1: D-Erythro-N-palmityol sphingosyl-1-0 Carbamoyl Spermine, CCS (Biolab Ltd., Jerusalem)

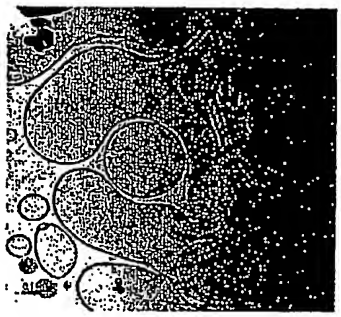


Fig. 2:Cryo-tem image of CCS dispersion

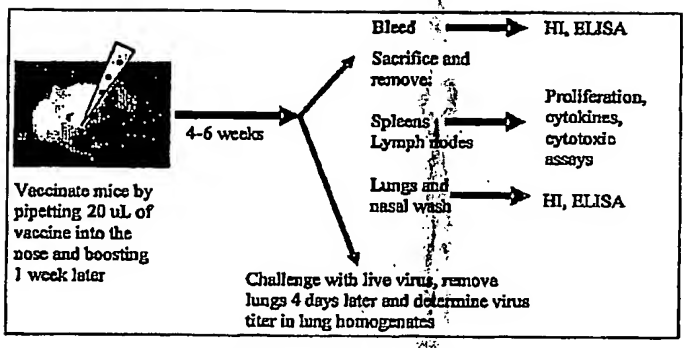


Fig. 3 : Experimental design

Some of these formulations also included "helper" lipids (dioleoyl phosphatidyl ethanolamine - (DOPE) as a fusion inducer, and cholesterol (Chol), in addition to the cationic lipids [31, 36, 43, 44]). A simple and fast (5 min.) mixing procedure has been developed for efficient (>80%) entrapment/ association of protein antigens in these cationic liposomes/lipid assemblies as determined by floatation over D_2O buffer [45]. Several experiments in the influenza model and pilot experiments with hepatitis A, M. tuberculosis and C. botulinum toxoid vaccines indicated that some of these cationic lipid-based vaccine formulations, when administered i.n., are highly effective in evoking mucosal and systemic Th1+ Th2 responses that were up to 10,000 times greater than those obtained with the standard vaccine. Below are representative results obtained in the influenza and botulism models. The experimental protocol is shown above in Fig. 3.

C4 Summary of CCS-flu vaccine preclinical experience

In the influenza model, a commercial monovalent (or trivalent) vaccine (2-6 μg/dose) comprising the virus surface antigens hemagglutinin + neuraminidase (HN) was administered twice i.n. (spaced 1 week apart) to young (2 mo. old.) BALB/c mice, either alone, with cholera toxin (CT, 1μg) as an adjuvant, or incorporated in neutral, anionic or cationic liposomes/lipid assemblies +/- CpG-ODN (10 μg) as an adjuvant.

C 4.1 Anti Influenza immune responses in young mice after i.n. monovalent influenza vaccination

The anti-influenza humoral and cellular (IFNγ) responses following i.n. vaccination with the HN antigens delivered by the various liposomal formulations were assessed. As can be seen in Figs. 4-6, only the cationic vaccine formulations comprising DOTAP, DMTAP, and especially those based on CCS (groups 9-11), administered i.n., were

highly efficacious, inducing high levels of HI (hemagglutination inhibition), IgG1, IgG2a and IgA antibodies in the serum, nasal wash and lungs, as well as IFNγ production by splenocytes. The most efficient was the CCS formulation (group 11), being even superior to the standard vaccine co-administered with the "gold standard" mucosal adjuvant cholera toxin (CT) (group 12) and to a liposomal (DMPC/DMPG) vaccine containing CpG-ODN as an adjuvant (group 5). Especially impressive are the high levels of lung IgA Abs, which are considered the most effective in protecting the respiratory tract.

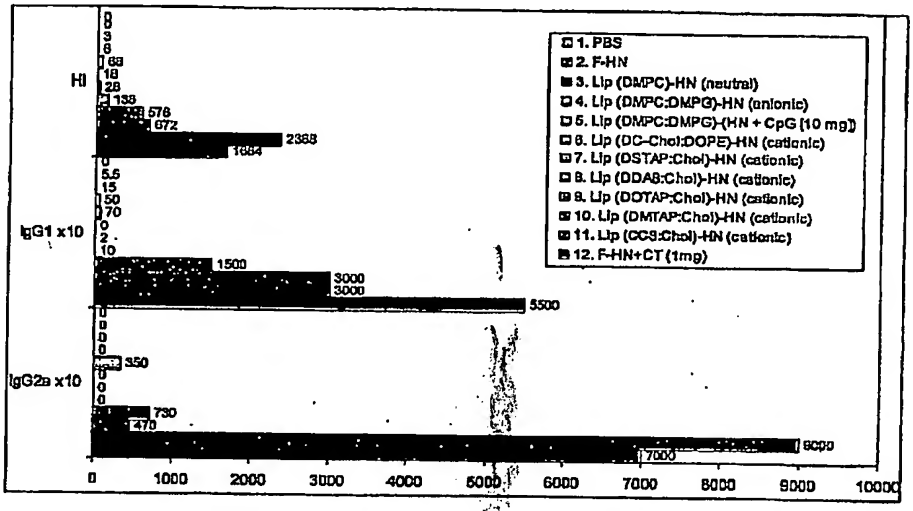


Fig. 4: Serum levels of HI, IgG1 and IgG2a antibodies following intranasal vaccination of young (2 mo. old) BALB/c mice with HN-loaded neutral, anionic and cationic liposomes

The subunit vaccine (3μg HN derived from A/New Caledonia (H1N1)) was administered i.n. (10 μl per nare) on days 0 and 7. All liposomes/lipid assemblies (Lip) were prepared at a lipid/protein w/w ratio of 300/1; the molar ratio of cationic lipid/cholesterol (Chol) or DOPE (groups 5-10) was 3/2. Serum HI titer was tested 4 weeks after the second vaccine dose on individual samples starting at 1/10 dilution; the data show the mean. Antigen-specific isotypes were determined by ELISA on pooled samples starting at 1/20 dilution; the data show the mean titers, determined at the sample dilution yielding OD=0.2 above the control (antigen + normal mouse serum). F-HN, free antigen. 0 denotes <10 for HI and <20 for isotypes. Groups 3,4 vs. groups 8-11 $p \leq 0.02$ (*t*-test).

Antigen-specific isotypes were determined by ELISA on pooled lung homogenates and nasal wash samples starting at 1/20 dilution 6 weeks after the second vaccine dose on individual samples. For details see Fig. 4.

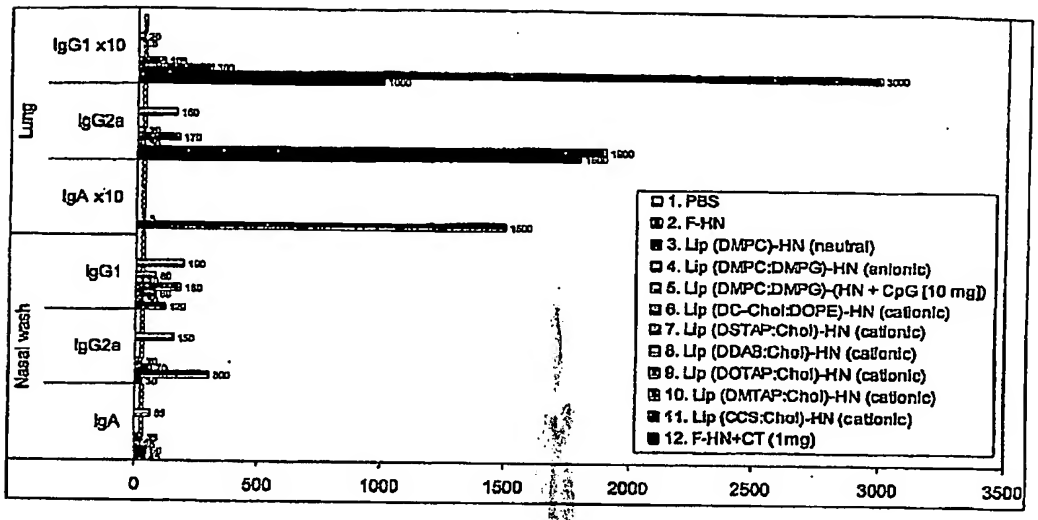


Fig. 5: Lung and nasal wash levels of IgG1, IgG2a and IgA antibodies following intranasal vaccination of young (2 mo. old) BALB/c mice with HN-loaded neutral, anionic and cationic liposomes

Spleen cells were incubated with the HN subunit preparation (10 µg/ml hemagglutinin) for 3 days. IFNγ levels in cell cultures without antigen were subtracted. In all groups, IL-4 levels were <20 pg/ml.

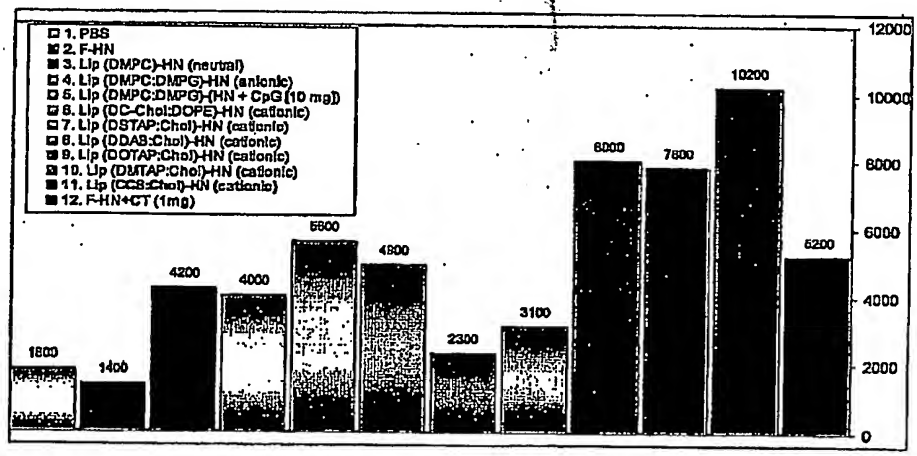


Fig. 6: Spleen IFNγ levels (pg/ml)

C 4.2 Anti influenza immune responses in aged mice after i.n. influenza monovalent vaccination

The immunogenicity of the CCS-influenza vaccine was also evaluated in aged (18 mo.) mice and, as opposed to zero activity of the commercial vaccine, the CCS-flu vaccine evoked high levels of serum HI and IgG2a Abs, and lung IgG2a and IgA Abs as can be seen in Fig. 7.

Mice were immunized twice i.n. (days 0, 7) or once i.m. (day 0) using 2 µg and 1 µg, respectively, of a subunit (HN) vaccine derived from A/Panama (H3N2) virus. The lipid assemblies were composed of CCS/cholesterol (3/2 mole ratio) and the lipid/HN w/w ratio was 200/1. The data show mean titers. Sera were tested at 4 weeks and lung homogenates at 6 weeks post vaccination. 0=<10.

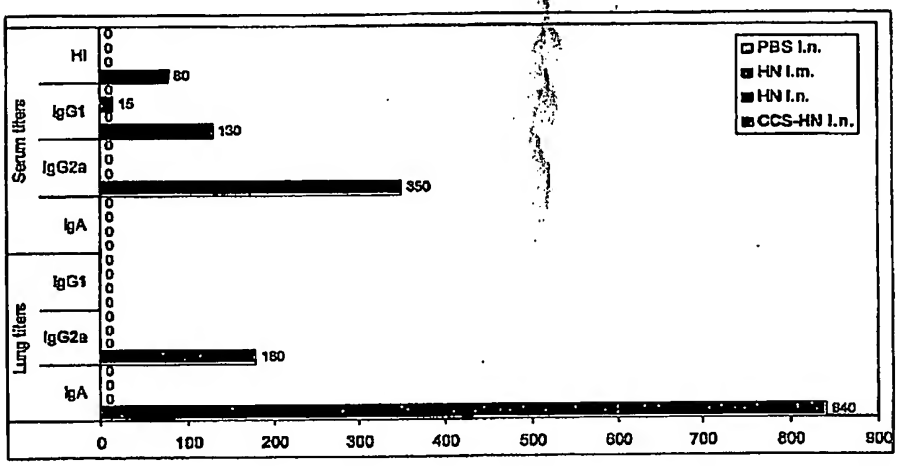


Fig. 7: Systemic and local humoral responses of aged (18 mo. old) C57BL/6 mice following intramuscular or intranasal

C 4.3 Anti-influenza CCS-vaccine induces cellular responses

The CCS formulation also induces cellular anti-influenza responses (proliferation, cytotoxicity, IFNγ) (Table 1). Interestingly, whereas maximum humoral responses were obtained at lipid/protein w/w ratio of 100-300/1 (data not shown), cellular responses were greater at 30-100/1. Therefore, in the proposed studies we will test humoral and cellular responses following vaccination with CCS formulations of varying lipid/protein ratios. It was also found that even simple mixing of the soluble antigen with preformed empty CCS lipid assemblies in an aqueous medium results in efficient antigen-CCS association (>50%) and very efficacious vaccination (group 7, Table 1), thus making the self-application of the vaccine easy and attractive.

No.	Vaccine[a]	Lipid/HN w/w ratio	% Cytotoxicity (Mean) P815+peptide	P815	Proliferation Δcpm (mean)	IFN (pg/ml)
1	PBS		6[c]/5[b]	4[c]/4[d]	7009/3493[d]	1900
2	F-HN		8/4	5/4	7704/6959	4500
3	CCS-HN	300/1	4/0	2/1	20370/17624	8000
4		100/1	21/13	7/6	24869/22014	8250
5		50/1	6/5	3/3	20984/13010	10650
6		30/1	8/7	5/4	11510/9699	3500
7	F-HN+Empty CCS	300/1	16/11	8/6	19269/15792	4100
8	F-HN+CT (1µg)		10/5	9/3	28580/20858	22800

Table 1: Induction of cellular responses by free and CCS-associated HN (H1N1) administered i.n. to young BALB/c mice

[a] Mice were immunized on days 0 and 7. Splenocytes were harvested 6 weeks after the second vaccine dose. All lipid (CCS) formulations (groups 3-7) were made with Chol at a lipid/Chol 3/2 mole ratio. In groups 3-6 the antigen was encapsulated in the lipid assemblies; in group 7 the free antigen was admixed with preformed empty lipid assemblies.
[b] Measured in a 4h ^{51}Cr release assay at effector/target cell ratios of 100/1[c] and 20/1[d]. P815

were pulsed with the HA2 189-199 peptide.
[e] Splenocytes were incubat d with 5 μg[f] or 0.5 μg[g] antigen per well for 72 h then pulsed with ^{3}H-thymidine for 16 h.
[h] Cells were cultured for 72h +/- antigen (5 μg/well). IFNγ levels in supernatants obtained from splenocytes cultured without antigen were subtracted.

In an attempt to further potentiate the response, we added CpG-ODN, known to be an efficient mucosal adjuvant (Figs. 4, 5 and [14, 19]), free and lipid-associated, to the CCS vaccine formulation. CpG-ODN (ODN 1018) formulated in the cationic lipids DMTAP, DOTAP and CCS demonstrated a much stronger (x 5-10 times) adjuvant activity than CpG-ODN entrapped in the non-cationic liposomes (DMPC, DMPC/DMPG - data not shown [19]). However, the addition of liposomal CpG-ODN to the CCS-based influenza vaccine had no beneficial effect on the humoral response (data not shown). Thus, CCS-based vaccines may not require an exogenous adjuvant for efficacy (at least when 2 i.n. administrations are used), which is advantageous with regard to potential adjuvant-related adverse reactions.

C 4.1 CCS trivalent flu vaccine is highly efficacious against all three viral strains

The CCS formulation is also highly efficient for a trivalent flu vaccine following i.n. and i.m. administration as is demonstrated in Fig. 8.

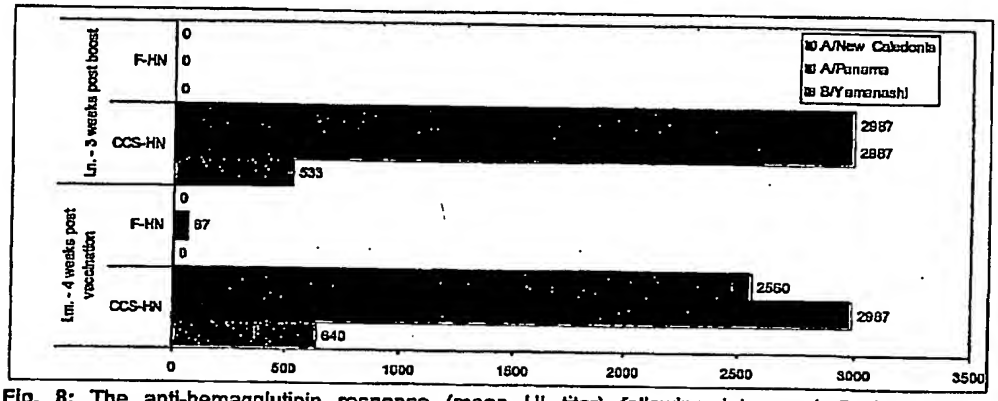


Fig. 8: The anti-hemagglutinin response (mean HI titer) following intranasal (i.n.) and intramuscular (i.m.) vaccination of young BALB/c mice (2 months) with a trivalent split influenza vaccine.

F-HN - the standard commercial vaccine;
CCS-HN - vaccine formulated with CCS:Cholesterol (3:2 mole ratio), lipid:protein w/w ratio 300:1.
0 denotes HI titer <20
SPF Balb/c female mice aged 9-10 weeks, n=3, were vaccinated either i.m. (x1) or i.n. (x2, spaced 1 week apart) with formulations based on the Fluvirin® 2003/2004 commercial split vaccine which was composed of -
A/New Caledonia/20/99 (H1N1)-like strain - 15 ug hemagglutinin/0.5 mL, (A/New Caledonia/20/99 IVR-116)
A/Moscow/10/99 (H3N2)-like strain - 15 ug hemagglutinin/0.5 mL, (A/Panama/2007/99 RESVIR 17)
B/Hong Kong/330/2001-like strain - 15 ug hemagglutinin/0.5 mL, (B/Shangdong/7/97)
The vaccine was concentrated x8 using a Speedvac concentrator (Eppendorf). For the i.m. vaccination, 0.5 ug HN/mouse of each viral strain (1.5 ug total) was injected i.m. in a volume of 50 uL. For the i.n. vaccination, 2 ug HN/mouse of each viral strain (6 ug total) was instilled i.n. in a volume of 10 uL/nare.

C 4.5 Summary of humoral, cellular and protective immunity evoked with the CCS-flu vaccine in young mice vaccinated i.n. or i.m.

Table 2 shows the humoral and cellular responses, and protective immunity to influenza virus challenge, following 1 i.m. and 1 or 2 i.n. administrations, using the free antigen (F-HN) or the cationic lipid-based vaccines. Whereas the i.m. lipid-based (but not the free) vaccines produced a good serum response (groups 5, 8, 11), only the

cationic formulations (groups 7, 10, 13), and in particular the CCS, administered x 2 i.n., elicited both high serum and mucosal responses, especially IgA, as well as impressive protective immunity. As in the previous experiment (Figs. 4-6), the CCS formulation (group 13) was much more efficient than the standard vaccine co-injected with CT (group 14), particularly for the serum, lung and nasal IgA Abs. The low efficacy of a single i.n. dose needs further study, which is included in our research plan (see Section D1, D4).

No.	Vaccine[a] (n=5)	Route	Serum (n=10) HI	IgG1	IgG2a	IgA	Lung (n=5) HI	IgG1	IgG2a	IgA	Nasal (n=5) IgA	Spleen (n=5) Δcpm (mean)[b]	IFNγ (pg/ml)[c]	Lung (n=5) Virus titer (log 10)[d]
1	PBS		0	0	0	0	0	0	0	0	0	1641	320	7
2	F-HN	i.m. x 1	60±37 (70)	1000	40	0	0	80	0	0	0	1909	410	4
3		i.n. x 1	0	0	0	0	0	0	0	0	0	669	0	ND
4		i.n. x 2	0	55	0	0	0	70	20	0	0	2813	220	5
5	DOTAP/Chol-HN	i.m. x 1	424±141 (100)	21000	5500	0	40	900	500	0	0	3452	3200	0
6		i.n. x 1	40±28 (50)	450	80	0	0	50	20	0	0	482	1900	ND
7		i.n. x 2	409±172 (100)	25000	1300	60	120	10000	1000	350	45	8391	3300	0
8	DMTAP/Chol-HN	i.m. x 1	768±211 (100)	24000	8000	0	20	900	150	0	0	5632	0	1
9		i.n. x 1	10±10 (0)	300	60	0	0	35	20	0	0	1277	0	ND
10		i.n. x 2	532±763(100)	10500	380	50	240	20000	700	2200	60	7331	3150	0
11	CCS/Chol-HN	i.m. x 1	864±1100 (100)	25000	10000	0	60	3500	900	0	0	6196	5750	0
12		i.n. x 1	34±50 (20)	1000	30	0	0	120	0	35	0	1705	7100	ND
13		i.n. x 2	2289±1578 (100)	25000	20000	400	360	30000	5000	20000	120	4912	15500	0
14	F-HN+CT (1ug)	i.n. x 2	756±650 (100)	21000	15000	20	240	22000	2500	1800	50	1933	5650	0

Table 2: Induction of humoral and cellular responses and protective immunity by free and HN-loaded cationic liposomes / lipid assemblies administered i.m. or i.n.

[a] BALB/c mice were immunized using 1 and 2 μg HN/dose (A/New Caledonia, H1N1) administered once i.m. (in 30 μl) or once or twice (spread 1 week apart) i.n. (10 μl/nare), respectively. All formulations were prepared at a lipid/HN w/w ratio of 300/1 with Chol at 3/2 mole ratio. Sera were tested separately (HI) or pooled (Elisa) at 4 weeks, and lung, nasal and spleen responses and protective immunity (all pooled) at 6 weeks post-vaccination. In parentheses, % seroconversion (HI titer >40). Isotype titers were determined as described in Figs 4-5.

[b] Δcpm = (mean cpm with antigen, 2.5 μg/well) – (mean cpm without antigen).

[c] IFNγ levels produced by spleen cells incubated alone were subtracted. 0 denotes <20 pg/ml.

[d] Tested 4 days after virus infection. ND, not done.

20

C 4.6 The CCS formulation is also effective for an anti-*C. Botulinum* intranasal vaccine.

The results of a pilot experiment with *C. botulinum* toxoid is demonstrated in Fig. 9. Here we show the superiority of the CCS-toxoid formulation over the standard vaccine or the vaccine supplemented with CT following i.n. instillation, particularly with regard to the IgA levels in the small intestine and feces. Such Abs are expected to neutralize the toxin upon oral exposure. Mice immunized i.n. with the vaccine alone or in combination with CT did not produce IgA.

Mice (n=10) were immunized on days 0, 7 with 0.4 µg/dose (20 µL) of a commercial toxoid (Uruguay, alum free). Pool d samples were tested by ELISA 1 month post.vaccination. Cholera toxin (CT, 1 µg/dose) was used as a mucosal adjuvant. The values are mean antibody titers determined as the highest sample dilution yielding 0.2 OD above the control (antigen + normal mouse serum).

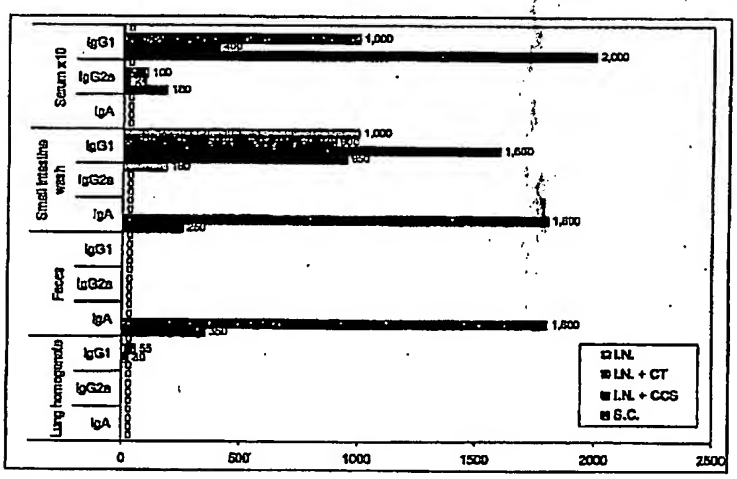
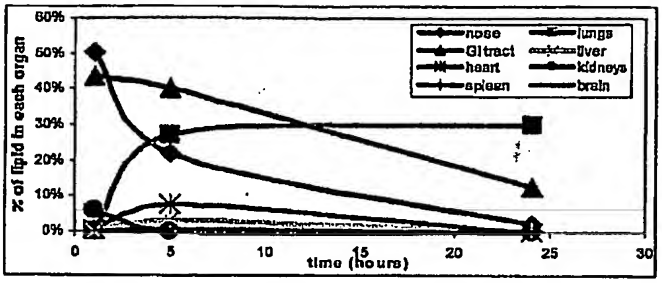


Fig. 9: The local and systemic humoral response of young (2 mo. old) BALB/c mice vaccinated i.n. or s.c. with free or CCS-associated *Clostridium botulinum* toxoid

C 4.7 Biodistribution

A potential hazard with i.n. vaccines is the migration of the vaccine components into the CNS [12a]. In a pilot experiment in which the CCS/Chol-HN was fluorescently labeled (lipid only), high levels of the marked lipid were found in the nose, lungs and GI tract up to 24 h after i.n. administration. The lipid was not traced in the brain (Figure 10). Further, more detailed studies are needed to complete the biodistribution, including those in which the antigen will also be labeled, and the fluorescent components will be replaced with radioactive ones in order to avoid the possibility of fluorophore induced artifacts.

Fluorescently labeled CCS loaded with the influenza vaccine (HN) were administered intranasally to mice. Lipid levels (according to fluorescence) were measured in the various tissues at 1, 5 and 24 hours after administration. There is sustained retention of the lipid in the nose, lung and GI tract (the relevant target organs), while there is no lipid accumulation in the brain, heart or kidneys.



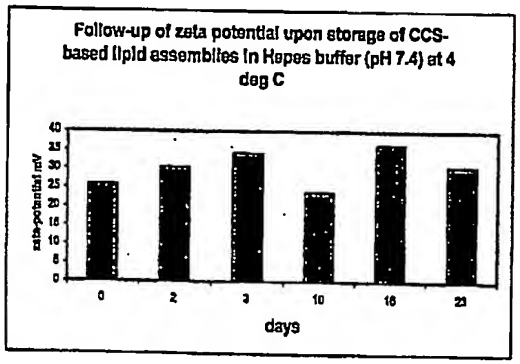
* 100% recovery up to 5 hours

Fig. 10: Lipid biodistribution in mice after intranasal administration of CCS-based influenza vaccination

C 4.8 CCS stability

We conducted a preliminary short-term (3 weeks) stability evaluation of the CCS formulation (without the influenza virus HN antigens), where the formulation was stored as aqueous dispersion at 4°C, pH 7.4. As shown in Fig. 11, both the zeta potential (positive charge) and the chemical stability (amine content) remained stable throughout the observation period. Lyophilized CCS was stable for >1 y. Further, longer-term stability tests of both CCS alone and CCS+HN antigens in dried form and in aqueous dispersions are planned in the proposed studies.

The positive charge (zeta potential) of CCS remains unchanged over >3 weeks



The chemical structure (primary amino groups) of CCS is stable over >3 weeks

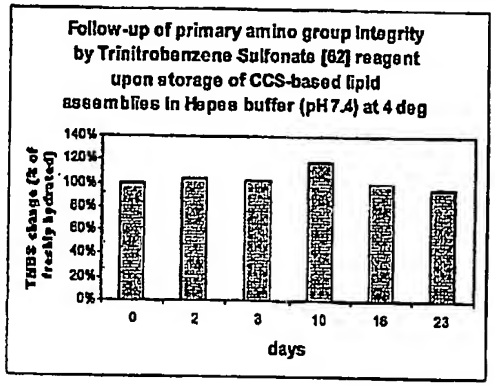


Fig. 11: Stability of the CCS formulations

C4.9 T xicology

Toxicity (local, systemic) is a major concern with both i.m. and i.n. vaccines and therefore we performed a pilot toxicity study. Cationic lipid formulations (DMTAP, DOTAP, CCS-based) loaded with the influenza antigens hemagglutinin + neuraminidase (HN) were administered i.n. (twice, spaced 1 week apart) to mice (n=4/group), and blood counts (total, differential), blood chemistry and histological examination (nose, lung sections) were performed 72 h. later. The mice showed no apparent signs of any toxicity. Blood counts and blood chemistry were within the normal range, and, as expected, minimal-mild inflammatory response was seen in the nose and lungs of mice treated with the cationic formulations. A similar, albeit less pronounced, inflammatory response was also seen in some mice treated with saline alone or with the non-encapsulated antigen. More screening studies followed by more detailed studies of the optimal vaccine formulations are required.

Summary of preliminary studies with the CCS-flu vaccine

The preliminary studies demonstrate that the CCS-based flu vaccine is highly efficacious upon i.n. and i.m. administration to mice in inducing robust local (i.n. only) and systemic humoral and cellular responses, as well as protective immunity to viral challenge. Vaccination with the CCS-flu vaccine is associated with only minor local inflammatory responses with no systemic toxicity. Preliminary biodistribution studies indicate retention of the vaccine in the respiratory tract (nose, lungs) for several hours, with no appearance in the brain. The CCS formulation is stable for at least 3 weeks upon storage as aqueous dispersion at 4°C, and for at least a year in the dry state.